EXHIBIT 1

Monoclonal antibody screening of a phage-displayed random peptide library reveals mimotopes of chemokine receptor CCR5: implications for the tertiary structure of the receptor and for an N-terminal binding site for HIV-1 gp120

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The chemokine receptor CCR5 contains seven transmembrane-spanning domains. It binds chemokines and acts as co-receptor for macrophage (m)-tropic (or R5) strains of HIV-1. Monoclonal antibodies (mAb) to CCR5, 3A9 and 5C7, were used for biopanning a nonapeptide cysteine (C)-constrained phage-displayed random peptide library to ascertain contact residues and define tertiary structures of possible epitopes on CCR5. Reactivity of antibodies with phagotopes was established by enzyme-linked immunosorbent assay (ELISA). mAb 3A9 identified a phagotope C-HASIYDFGS-C (3A9/1), and 5C7 most frequently identified C-PHWLRDLRV-C (5C7/1). Corresponding peptides were synthesized. Phagotopes and synthetic peptides reacted in ELISA with corresponding antibodies and synthetic peptides inhibited antibody binding to the phagotopes. Reactivity by immunofluorescence of 3A9 with CCR5 was strongly inhibited by the corresponding peptide. Both mAb 3A9 and 5C7 reacted similarly with phagotopes and the corresponding peptide selected by the alternative mAb. The sequences of peptide inserts of phagotopes could be aligned as mimotopes of the sequence of CCR5. For phage 3A9/1, the motif SIYD aligned to residues at the N terminus and FG to residues on the first extracellular loop; for 5C7/1, residues at the N terminus, first extracellular loop, and possibly the third extracellular loop could be aligned and so would contribute to the mimotope. The synthetic peptides corresponding to the isolated phagotopes showed a CD4-dependent reactivity with gp120 of a primary, m-tropic HIV-1 isolate. Thus reactivity of antibodies raised to CCR5 against phage-displayed peptides defined mimotopes that reflect binding sites for these antibodies and reveal a part of the gp120 binding sites on CCR5.

Key words: Phage-displayed peptide library / Mimotope / Chemokine receptor CCR5 / HIV-1 / gp120

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1 Introduction

Chemokines are chemotactic cytokines that are important for leukocyte trafficking [1–3]. They are secreted by various cell types and engage cognate receptors that are located on macrophages, T lymphocytes and other leukocytes. According to the classification of their specific ligands four classes of chemokine receptors are known, including XC-chemokine receptors, CC-chemokine receptors, CXC-chemokine receptors, CX_3C-chemokine receptors and a number of orphan chemokine receptor-like proteins [4–6]. Knowledge of the structure of chemokine receptors is incomplete, but they are proposed to have seven transmembrane-spanning domains. The tertiary structure of a related receptor, bacteriorhodopsin, has been solved [7]; the model possesses seven trans-

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Abbreviations: CCR: CC chemokine receptor **CXCR:** CXC chemokine receptor **RP:** Reverse phase **m-tropic:** Macrophage-tropic **MS:** Mass spectroscopy **s:** Soluble

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EXHIBIT I

membrane helices arranged in a circular fashion. A similar tertiary structure is proposed for chemokine receptors. Interest in chemokine receptors has been increased by the recognition that these also serve as co-receptors for HIV-1 entry into cells [6, 8–18]. At present as many as 13 human chemokine receptors have been identified that function as co-receptors *in vitro*, and this number is likely to increase, although the relevance *in vivo* of most of them is questionable. In fact, only CCR5 and CXCR4, and to some degree CCR3 [16], may be relevant for viral entry *in vivo*.

HIV-1 isolates are macrophage (m)-tropic, T cell (T)-tropic or dual-tropic according to their specificity for host cells. CCR5 is the major co-receptor for m-tropic HIV-1 isolates, and CXCR4 for T-tropic isolates. However, as the classification m-tropic and T-tropic is imprecise, isolates have been renamed according to their co-receptor usage; isolates that use CCR5 or CXCR4 are called R5 or XR4 isolates, respectively [17]. Most primary isolates of various HIV-1 subtypes tested have been identified either as R5 or R5X4 virus, and none could use any of the other receptors for viral entry mentioned above [19, 20]. Additionally, there is evidence that CCR5 is important for primary infection *in vivo*. This includes the cell type expression of CCR5 [21] and the fact that HIV-1 isolates in early stages of infection are mainly R5 viruses [22]. Notably individuals with a mutant non-functional CCR5 are highly resistant to HIV infection [23, 24]. Infection *in vitro* of $CD4^+$ cells by R5 isolates is inhibited by the natural ligands for CCR5 [25], by receptor antagonists [26], and also by murine mAb, including mAb 3A9 and 5C7 that were raised against cells transfected with CCR5 [21]. Both mAb inhibit m-tropic HIV-1 infection without affecting chemokine binding. Previous studies with mAb to CCR5 and with cells transfected with chimeric forms of CCR5 and CCR2b have indicated that there is an epitope near the N terminus of CCR5 for both 3A9 and 5C7 [27], but precise binding sites for the mAb have not been identified.

We therefore used a phage-displayed random nonapeptide library to ascertain the contact residues for mAb to CCR5. We describe the isolation of phage clones (phagotopes) with highly homologous peptide sequences that do not have a direct linear correspondence with the sequence of the receptor and therefore define residues of a conformational epitope, *i.e.* a mimotope. Of two identified mimotopes, one defined contact residues on the N terminus and the first extracellular loop of the receptor, and another residues at the N terminus, first extracellular loop and a likely site on the third extracellular loop.

2 Results

2.1 Peptide sequences from phage-displayed libraries

For mAb 3A9, 26 phage clones were isolated after five rounds of biopanning. All clones were tested by capture ELISA for reactivity with mAb 3A9 and 23 of 26 clones showed strong reactivity. After sequencing, 19 non-americ peptide inserts could be identified. Of these 19 clones, 18 displayed an identical insert, C-HASIYDFGS-C, designated 3A9/1. One clone, 3A9/8, displayed the insert C-VYALIMPPL-C. The N- and C-terminal residues represent the cysteine "constraints" (see Sect. 4.2). For mAb 5C7, after three rounds of biopanning, 64 phage clones were isolated and tested by capture ELISA for reactivity with mAb 5C7. Nineteen clones showed very strong reactivity and sequencing revealed five different phagotypes (Table 1); the sequence of the peptide insert in the most frequently (15/19) isolated phagotope, 5C7/1, was C-PHWLRDLRV-C. The remaining four sequences (Table 1) were each isolated once.

2.2 Reactivity of mAb with phage clones and synthetic peptides by ELISA

Phage clones were tested in a direct ELISA using the selecting mAb. As expected, the 18 phage clones showing the same insert (3A9/1) gave an identically high reactivity. The reactivity with phage clone 3A9/8 was slightly lower. For phages selected with mAb 5C7, the 15 clones (5C7/1) likewise showed high reactivity with mAb 5C7. The reactivity of the other four phagotopes was slightly lower (Table 1). Phage clones without an insert and those having a stop codon in the region coding for the insert served as negative controls.

Two peptides, P3A9/1c and P5C7/1c, corresponding to the sequence of phagotopes isolated with mAb 3A9 or 5C7, were synthesized and oxidized to form a disulfide bond between the two terminal cysteine residues since the constrained peptide insert is expected to be cyclic in the coat protein of the phage. The structure of the peptides was confirmed by mass spectroscopy (MS) (data not shown). The two mAb reacted with the corresponding synthetic cyclic peptides by ELISA in a dose-dependent manner (Fig. 1) although the reactivity was much less than that obtained using the phage immobilized on the plate. Both the cyclic and linear control peptides gave substantially lower degrees of reactivity (Fig. 1). The reactivity of P5C7/1c was almost tenfold greater than the reactivity of P3A9/1c with mAb 3A9; the reactivity of P3A9/1c with mAb 3A9 was significantly greater than that of the control peptides only at the high-

Table 1. Sequences of phage clones isolated by biopanning, frequency and reactivity by ELISA

Name of phagotope	Sequence	Number of times selected	A[a]
mAb 3A9 selected			
3A9/1	C-HASIYDFGS-C	18	2.7[b]
3A9/8	C-VYALIMPPL-C	1	2.6
mAb 5C7 selected			
5C7/1	C-PHWLRDLRV-C	15	2.4[c]
5C7/8	C-LPPSYCFGS-C	1	2.4
5C7/9	C-PPVFGTFTS-C	1	2.2
5C7/54	C-YGPFSRASY-C	1	1.6
5C7/58	C-MPPSMTSVS-C	1	1.8

a) A values indicate units (415 nm) measured at 15 min, after subtraction of background.
b) All 18 phagotopes with this sequence were highly reactive (A > 2.5).
c) All 15 phagotopes with this sequence were highly reactive (A > 2.4).

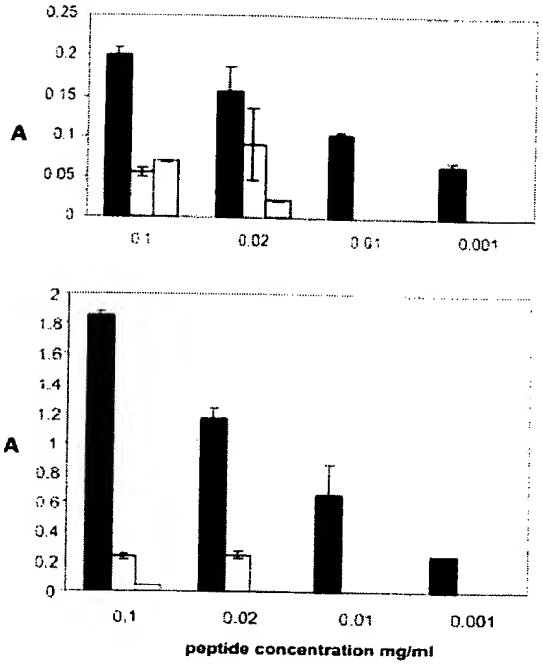

Fig. 1. mAb 3A9 and 5C7 bind synthetic peptides. The synthetic peptides P3A9/1c (upper) and P5C7/1c (lower) (black bars) were immobilized on microtiter plates and tested by ELISA with 5 μg/ml mAb 3A9 (upper) and mAb 5C7 (lower). Absorbance (y axis) was measured at 415 nm after 30 min. Columns show means (n = 2) and error bars represent ± 1 SD of the mean. The control peptides PCII-C1c (white bars) and PGAD1 (gray bars), tested at 0.1 and 0.02 mg/ml, were minimally reactive.

est peptide concentration (0.1 mg/ml), possibly because contact residues for binding of the peptide to mAb 3A9 were masked during immobilization of the peptide on plastic. The synthetic peptides in their linear forms, P3A9/1 and P5C7/1, were tested in the same way. The linear peptides showed reactivity with the mAb in a dose-dependent manner but to a much lesser degree than that of the cyclic peptides (data not shown). The C residues of the linear form were protected to avoid spontaneous oxidation.

Inhibition ELISA were performed to test whether the peptides inhibited the binding of mAb to the phage displaying the peptide insert of interest. Clones 3A9/1 and 5C7/1 were chosen for inhibition studies since they showed the strongest reactivity with the mAb. The mean absorbance observed in 12 wells without inhibitor was used as zero inhibition, and significant inhibition was assessed by an absorbance less than mean minus 2 SD for the control wells. This represented > 5 % inhibition for mAb 3A9, and > 8 % for 5C7. Peptides P3A9/1c and P5C7/1c inhibited binding of the mAb 3A9 and 5C7 to the phage clones 3A9/1 and 5C7/1, respectively, in a dose-dependent manner. Reactivity of mAb 3A9 with phage clone 3A9/1 was inhibited up to 44 % at 100 μg/ml P3A9c, and reactivity of mAb 5C7 with phage clone 5C7/1 was inhibited up to 55 % at 100 μg/ml P5C7c (Fig. 2). It was not possible to test the peptides at concentrations > 0.1 mg/ml, and complete inhibition with the peptides could not be demonstrated because of the very strong reactivity of the mAb with the corresponding phagotopes (ELISA A > 2 at 15 min). However, there was no evidence that the inhibition had reached a plateau at 0.1 mg/ml, so that 100 % inhibition would likely have been achieved if higher peptide concentrations had been attainable. With the control peptide PGAD1 at any concentration, the maximum inhibition obtained was 3 % for either mAb (Fig. 2).

The ability of each mAb to bind to phagotopes selected by the alternative mAb was tested by direct ELISA. Both 3A9 and 5C7 treated similarly with the phagotope most frequently selected by the alternative mAb (Fig. 3), and this was confirmed by using the synthetic peptides as antigen in similar assays (Fig. 3, inset).

The affinity of binding of mAb with the selected and alternative peptides was 19 nM and 18 nM for 3A9 and 13 nM and 23 nM for 5C7 as determined by Biocore (see Sect. 4.5.4).

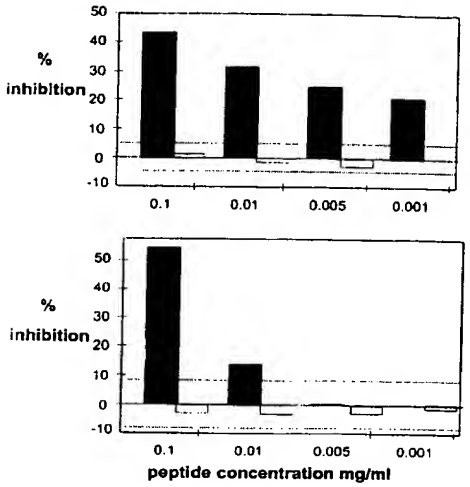

Fig. 2. Synthetic peptides inhibit binding of mAb to phage clones. The phage clones 3A9/1 (upper) and 5C7/1 (lower) were immobilized on microtiter plates and tested by ELISA with 5 μg/ml mAb 3A9 (upper) and mAb 5C7 (lower). The synthetic peptides P3A9/1c (upper) and P5C7/1c (lower) (black bars) were added to the mAb at different concentrations to demonstrate inhibition by synthetic peptide of interaction of mAb with phage clones. Absorbance was measured at 415 nm after 30 min. There was not inhibition with the control peptide PGAD1 (white bars). Significant inhibition was based on mean A ± 2 SD for 12 control wells without an inhibitor.

2.3 Inhibition of mAb binding to cell surface-expressed CCR5

mAb 3A9 reacted strongly with CCR5-transfected L1.2 cells, as reported [21]. The peptide P3A9/1c inhibited staining of cells with mAb 3A9 over a range of concentrations, with a maximum of 61 % at a peptide concentration of 0.1 mg/ml compared with mAb 3A9 staining with no inhibiting peptide (Fig. 4). The inhibition from peptide concentrations of 0.01 mg/ml and higher was significant ($p < 0.00005$).

2.4 Synthetic peptides bind gp120 of primary isolate

Peptides B ~ P3A9/1 and B ~ P5C7/1 bound to epitopes on a clade B primary isolate of HIV-1 termed D117III [28]. The binding was CD4 dependent (Fig. 5). Both peptides gave reactivity only at the highest peptide concentration tested (100 μg/ml). For B ~ P3A9/1 reactivity was doubled in presence of 10 ng/ml soluble (s) CD4 compared with the control without sCD4. For

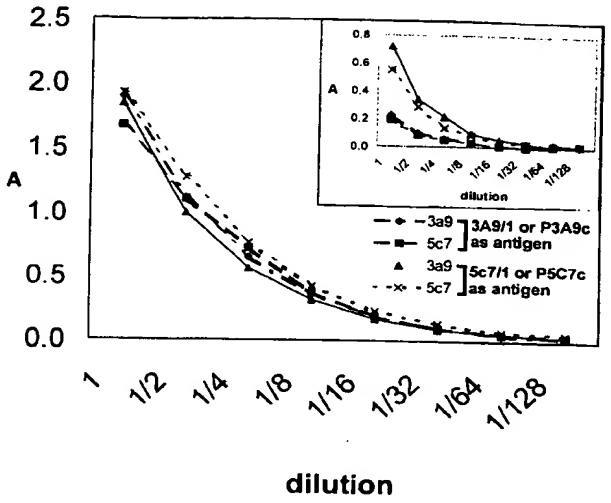

Fig. 3. mAb 3A9 and 5C7 bind to phagotopes selected by either antibody. ELISA ($A_{415\ nm}$, y axis) were performed as described for Figs. 1 and 2 using doubling dilutions of the mAb 3A9 and 5C7. Both 3A9 and 5C7 reacted equally well with the most frequently selected phagotopes 3A9/1 and 5C7/1. Similar results were also obtained (inset) using the synthetic peptides P3A9c and P5C7c

B ~ P5C7/1, addition of sCD4 resulted in a tenfold increase in reactivity. Comparing both peptides, B ~ P3A9/1 showed a remarkably higher reactivity with the virus isolate than B ~ P5C7/1.

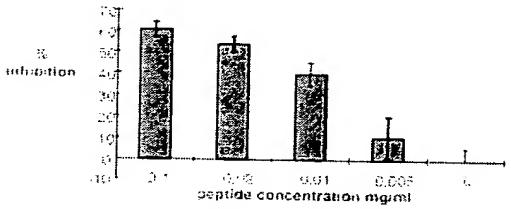

Fig. 4. Synthetic peptide P3A9/1c inhibits binding of mAb 3A9 to CCR5. Microscope slides were coated with the CCR5-expressing cell line L1.2 CCR5 and tested with 5 μg/ml mAb 3A9 by indirect immunofluorescence (see Sect. 4.6). The synthetic peptide P3A9c was added at concentrations of 0.001 to 0.1 mg to show inhibition of mAb interaction with the receptor. The emitted fluorescence was measured (see Sect. 4.6) and the mean fluorescence ($n \sim 65$ cells measured per peptide concentration) is shown as percent inhibition. Bars show 1 SEM.

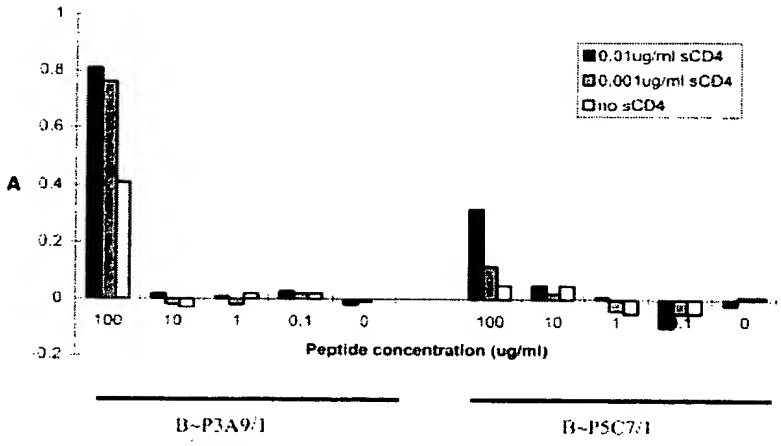

Fig. 5. Synthetic peptides bind CD4-induced epitopes on gp120. Primary HIV-1D117III was immobilized on microtiter plates; sCD4 and biotinylated peptides were added at the concentrations indicated. In parallel biotin attached to a peptide spacer was added at the same concentrations as for the negative control. The A (492 nm, y axis) observed with the negative control (maximal A with 100 μg/ml peptide: 0.031) was subtracted from the A measured for peptides B ~ P3A9/1 and B ~ P5C7/1. Columns show the mean of peptide samples minus the background.

3 Discussion

The screening of a nonameric phage-displayed random peptide library with mAb raised against the chemokine receptor CCR5 has identified two peptide inserts that react as phagotopes and also as linearized and cyclic synthetic peptides with the corresponding mAb. These peptides represent mimotopes indicative of the reactive sites for these antibodies. Previous studies using chimeric molecules identified a reactive site on CCR5 for mAb, including 3A9 and 5C7, located at the N terminus [27], but without identification of contact residues, and a site on the second extracellular loop was postulated, based on use of an mAb not included in this study. These data, and the reported sequence of CCR5 [29], allow the following sequence alignments. For the sequence isolated with mAb 3A9, C-HASIYDFGS-C, the residues SIYD can be aligned to residues 6 or 7, 9, 10 and 11 at the N terminus of CCR5, and the residues FG to residues 96 and 97 on the first extracellular loop (Fig. 6 a). These residues do not occur on the N terminus of the receptor CCR2b, consistent with the lack of reactivity of mAb 3A9 with a chimeric molecule displaying the N terminus of CCR2b on a CCR5 background [27]. On the other hand, the mimotope for 3A9 contained the motif FG which is shared by the first extracellular loop of CCR5 and CCR2b, and chimeric molecule containing the first extracellular loop of CCR2b on a CCR5 background displayed all identified contact residues, and hence was fully reactive with mAb 3A9. However, the identification of critical residues for mAb binding that might be shared by both receptors cannot be addressed by studies on chimeric molecules.

The mimotope for the other mAb, 5C7, represented similar regions of CCR5, together with a possible site on the third extracellular loop. For the prevalent sequence C-PHWLRDLRV-C isolated by mAb 5C7, there is alignment to residue 8(P) at the N terminus, and to residues 88(H) and 94(W) on the first extracellular loop, as for the 3A9-derived sequence (Fig. 6 b). A presumed motif, represented by the phagotope sequence DLR, could correspond to a sequence of CCR5, residues 274–276 (RLD), on the third extracellular loop but exhibited in reverse, assuming that the third extracellular loop has a location opposite to the first extracellular loop, as shown in Fig. 6 b. Reactivity of mAb 5C7 with the N terminus has already been shown [27]. The residues HW of the mimotope, representing parts of the first extracellular loop, are also present on CCR2b as are two of the three residues RLD on the third extracellular loop. Our conclusions are supported by the reactivity of synthetic peptides with sequences corresponding to those of the isolated phagotopes. Thus the peptides C-HASIYDFGS-C and C-PHWLRDLRV-C inhibited the strong reactivity of mAb 3A9 or 5C7 with the cognate phagotopes when tested by ELISA, and C-HASIYDFGS-C inhibited the binding of mAb 3A9 to cells transfected with a cDNA for CCR5 in studies by indirect immunofluorescence. Moreover, both of the mAb 3A9 and 5C7 reacted in ELISA with the phagotopes and the corresponding peptide selected by the alternative mAb, suggesting that the epitope recognized

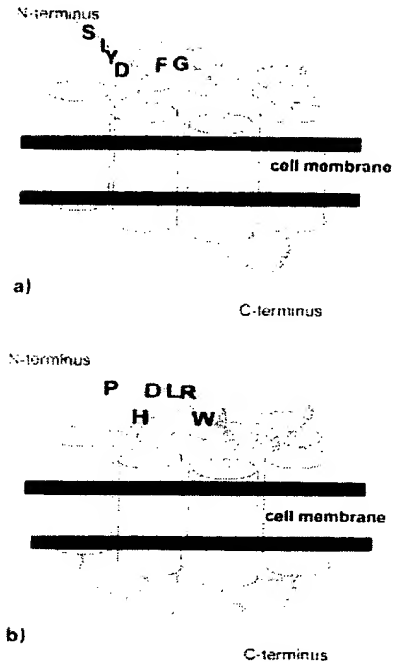

Fig. 6. Proposed contact residues on chemokine receptor CCR5. Contact residues and their presumed location are shown for (a) mAb 3A9 on the N terminus (SIYD) and the first extracellular loop (FG) and for (b) mAb 5C7 on the N terminus (P), the first extracellular loop (HW) and the third extracellular loop (DLR).

by 3A9 and 5C7 is similar. These observations are in line with the predicted structure of chemokine receptors, since a circular alignment of transmembrane α-helices would place the third extracellular loop opposite to the first extracellular loop, and create a conformational epitope from residues on the N terminus, and the first and third extracellular loop. Topographically the mimotopes for 3A9 and 5C7 involve similar regions of CCR5, as expected since both antibodies have been shown to react with the N terminus and have similar properties in that they inhibit CCR5-mediated entry of HIV-1 but do not affect interaction of chemokines with the receptor. The apparent reactivity of the mAb with the N terminus, the first extracellular and possibly the third extracellular loop, without affecting chemokine binding points to the second extracellular loop as a critical site for chemokine interaction, consistent with previous findings from studies on chimeric receptors [27].

Our data on epitope mapping for mAb to CCR5 are supported by a recent study by Lee et al. [30] who mapped 18 mAb to CCR5 on chimeric molecules and by alanine scan mutagenesis. They identified only a few antigenically dominant sites, with the indication that some of their mAb would react with epitopes similar to 3A9 and 5C7. The epitopes for 7 of the 18 mAb were mapped to the N terminus on chimeric molecules, and site-directed mutagenesis revealed binding residues for three mAb on the N terminus of CCR5 identical to those identified for the mAb of our study. These residues include S6/7, I9, Y10 and D11. Only one of the mAb (CTC8) in the study of Lee et al. [30] with these contact residues was tested for inhibition of chemokine binding, gp120 binding and cellular fusion. It showed effects similar to those of 3A9 and 5C7, but to a much lesser degree [21, 30]. This may account for the fact that CTC8 recognizes a linear epitope on the N terminus, whereas 3A9 and 5C7 recognize conformational epitopes. Another antibody, CTC12, had identical contact residues on the N terminus as 3A9, and also recognizes a conformational epitope, but this mAb was not tested in inhibition assays. Lee et al. [30] recognized the same limitations to the use of chimeric receptors for epitope mapping that we comment on above. Thus they could not exclude any involvement of additional residues for mAb binding that were conserved in the receptors used for generation of the chimeric molecules. They tried to obviate these limitations by using a large panel of different chimeras, yet all residues on the first extracellular loop, and some of those on the third extracellular loop that were identified in our study using 3A9 and 5C7, were in fact present in all of the chimeric molecules used by Lee et al. Thus these residues could not be examined as possible contact residues for mAb to CCR5. In another study Olson et al. [31] raised another six mAb to CCR5, and mapped epitopes by use of alanine scan; binding of five out of these six mAb required the same N-terminal residues which we identified for 3A9 and 5C7, together with other residues on the extracellular loops.

mAb 3A9 and 5C7 inhibit binding of the gp120 molecule of HIV-1 to CCR5, but it is not known whether this inhibition occurs by competition for an identical binding site, or by steric hindrance. Several groups have shown the importance of the N terminus and extracellular loops for cellular entry of R5 viruses [6, 32, 33]. The significance of single residues for coreceptor function has been addressed by N-terminal truncations and alanine replacements of single residues. Despite the large variation in contact residues according to cell type and HIV-1 strain, most of the contact residues for 3A9 and 5C7 identified in this study contributed to gp120 binding; thus residues 1–19 and 4–25 were identified as crucial for coreceptor function [34, 35]. Among these residues, coreceptor function was attributed to 6S [36], 10Y [35, 37, 38], 11D [35, 37, 39], with other residues defined for various isolates (reviewed in [33, 40]). There are no data available for residues 94W, 95D, 96F and 97G of the first extracellular loop. However, two further studies demon-

strated the importance of the third extracellular loop for viral entry in that residues 274R and 276D, which are part of our proposed epitope for 5C7, were involved in co-receptor function [41, 37].

These and our own data strongly implicate the usage of similar residues of CCR5 for mAb 3A9 and 5C7 contact and for binding of gp120. Therefore we tested the synthetic peptides that represent the mAb binding sites for reactivity with the primary isolate HIV-$1_{D117III}$. We could show that both synthetic peptides bind to CD4-induced epitopes on a primary HIV-1 isolate. The mimotope corresponding to mAb 3A9 gave better binding than did the 5C7 mimotope, as expected because the peptide P3A9/1 contains a motif of the Y-rich region of the N terminus. This region has a charge similar to that of the surface of mAb 17b that binds to CD4-induced epitopes on gp120 as resolved in the gp120 crystal structure [42, 43]. Therefore the Y-rich region would be expected to bind to a similar part of the CD4-induced epitopes.

Taking together the data on residues required for HIV-1 entry, and the fact that both of the synthetic peptides corresponding to reactive phagotopes show a similar reactivity with the mAb, but have a remarkably different reactivity with the HIV-$1_{D117III}$, we conclude that the N-terminal part of the epitopes for mAb 3A9 and 5C7, and gp120 on the other hand, are not identical but overlap closely. This applies to a greater extent to 3A9 than to 5C7.

In conclusion, we have used a phage-displayed peptide library to ascertain nonapeptide mimotopes on the chemokine receptor CCR5 for two mAb known to react with the receptor and to inhibit CCR5-dependent entry of HIV-1. These mimotopes represent a part of the gp120 binding site on CCR5. Our findings bear on the tertiary structure of the receptor, and could be relevant to the design of drugs that prevent access of HIV-1 to CCR5-expressing cells, yet spare the interaction of the receptor with its natural ligands.

4 Materials and methods

4.1 mAb

mAb 3A9 and 5C7 were generated by immunizing mice with a murine prelymphoma cell line transfected with CCR5 (L1.2 CCR5) and were derived as described previously [21].

4.2 Phage-displayed random peptide library

The phage library contained 1×10^7 random nonapeptide-encoding inserts, "constrained" by flanking cysteine residues, in the pVIII coat protein of phagemid pC89 [44]. For biopanning [45], 2.7×10^{12} phage particles and 40 μg mAb in PBS, pH 7.3, supplemented with 1 mg/ml BSA (PBS-BSA) were left overnight at 4 °C and phage-antibody complexes were isolated using magnetic beads coated with anti-mouse IgG (Chemicon, Temecula, CA). Bound phages were eluted with 1 mg/ml BSA in 0.2 M glycine-HCl, pH 2.2, neutralized using 1 M Tris-HCl, pH 9.1, propagated in *Escherichia coli* strain K91 with helper phage M13KO7 (Pharmacia, Uppsala, Sweden), and grown selectively with 100 μg/ml ampicillin and 70 μg/ml kanamycin. Bacteria were pelleted by two centrifugations for 15 min at 40000 × g. Phage particles were precipitated from the supernatant with 5 % PEG 6000 and 0.5 M NaCl on ice for 1 h and pelleted by centrifugation for 15 min at 10000 × g. The pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 8. Precipitation with PEG/NaCl was repeated and the final phage pellet was resuspended in PBS and used for further rounds of biopanning. After the first, second and fifth positive selection with tenfold decreasing concentrations of mAb to the lower limit of 0.4 μg/ml, negative selections were performed with magnetic beads in PBS-BSA but no mAb. Single phage clones thus isolated were amplified and purified.

4.3 DNA sequencing

Phage clones that showed strong reactivity in the capture ELISA (see below) were selected, and single-stranded DNA was extracted [46] and sequenced using the Sequenase Version 2.0 T7 DNA Sequencing kit (Amersham, Life Science, Cleveland, OH), with samples run on a 4.5 % acrylamide denaturing gel before exposure to film and development [46].

4.4 Peptide synthesis

Peptides that corresponded to the two inserts most frequently isolated by the mAb, termed P3A9/1 and P5C7/1, were synthesized on a PS3 Protein Technologies Automatic Peptide Synthesizer (Rainin Instrument Company, Woburn, MA) by solid phase chemistry [47] using Fmoc-protected amino acids (Auspep, Parkville, Victoria, Australia) with analysis and purification of the peptides by reverse phase (RP)-HPLC and electrospray MS. As the peptide inserts are flanked by cysteine residues, a cyclic structure may form in the phage coat protein. Therefore an aliquot of the peptides was deprotected and oxidized with iodine in 95 % acetic acid to form a disulfide bond between the flanking cysteine residues to give the cyclic peptides P3A9/1c and P5C7/1c. Linear and cyclic forms of the peptides were verified by RP-HPLX and MS.

For gp120 binding studies the two peptides were again generated as above, but attached to biotin by a βAβA-spacer to generate B ~ P3A9/1 and B ~ P5C7/1. The βAβA-spacer was also attached to biotin without any further peptide (B ~ only).

As controls we used two peptides, one cyclic and one linear, and corresponds to two inserts from phagotopes derived by screening the library with two irrelevant mAb. The cyclic peptide C-IAPKRHNSA-C, termed PCII-C1c, was derived by screening with the mAb CII-C1 raised against type II collagen [48], and the linear peptide LKIGDFPAG, termed PGAD1, was derived by screening with the mAb GAD1 raised against glutamate decarboxylase [49].

4.5 ELISA procedures and measurement of affinity

4.5.1 Capture ELISA

Ninety-six-well microtiter plates (Maxisorp, Nunc, Denmark) were coated with 1 μg mAb per well in 100 μl PBS and left overnight in a humidified box at 4 °C. Plates were blocked for 1 h at room temperature with 1 % skimmed milk powder in 0.05 % Tween-20 in PBS (MP/PBS-T) and washed three times with Tris-buffered saline, pH 7.4 and 0.05 % Tween-20 (TBS-T). Duplicate samples of 100 μl PBS containing 5 μl of the phage clone were added to the wells and held overnight at 4 °C. Wild-type phage (f1) was used as a negative control. The plates were blocked and washed before adding 100 μl of a 1:2000 dilution of anti-M13 antibody (Pharmacia) in MP/PBS-T. Bound phage was detected by adding horseradish peroxidase (HRP)-conjugated anti-sheep/goat antibodies (Silenus, Hawthorn, Australia) diluted 1/2000 in MP/PBS, and development with 0.5 mg/ml 2,2-azino-di-(3-ethyl-benzthiazoline 6 sulfonate) (ABTS; Diagnostic Chemicals, Charlottetown, Canada) in 0.03 M citric acid, 0.04 M Na_2HPO_4, pH 4 and 0.003 % H_2O_2. After 15, 30 and 60 min, the A at 415 nm was measured.

4.5.2 Direct ELISA

Microtiter plates were directly coated with 5 μl amplified phage in PBS, left overnight at 4 °C, washed and blocked, and mAb were added at 10 μg/ml in PBS and left for 4 h. For measurement of cross-reactivity of mAb with the alternatively selected phagotopes and corresponding synthetic peptides, doubling dilutions of the mAb starting from 5 μg/ml were used. The plates were exposed to anti-mouse HRP-conjugated antibody diluted 1:2000 for 2 h.

4.5.3 Inhibition ELISA

The capacity of synthetic peptides to inhibit the binding of mAb 3A9 to phagotopes was measured by coating microtiter plates with selected phage clones, and adding inhibitory peptides at 100, 10, 5 or 1 μg/ml to 100 μl mAb 3A9 or 5C7 at 5 μg/ml.

4.5.4 Peptide ELISA

Plates were coated with the synthetic peptide P3A9/1c or P5C7/1c at concentrations of 100, 20, 10, 5 or 1 μg/ml, or no peptide, in carbonate/bicarbonate buffer, pH 9.6, left overnight, washed and blocked with PBS containing 3 % BSA. As primary antibody, 100 μl mAb 3A9 for peptide P3A9/1c and mAb 5C7 for peptide P5C7/1c, at a dilution of 5 μg/ml in PBS with 1 % BSA, was added to the wells; peptide-antibody complexes were detected using HRP-conjugated anti-mouse antibody. For the cross-reactivity studies, plates were coated with 20 μg/ml of the synthetic peptide. Control peptides were tested at 100 and 20 μg/ml.

The affinity of binding of mAb with the selected and alternative peptides was determined using a Biacore. Peptides were coupled to a CM5 sensorchip via the terminal cysteines, and five dilutions of antibody ranging from 31.25 to 580 nM were passed over the surface using PBS with 0.005 % Tween-20 as running buffer. Data were analyzed using BIA evaluation 3.0 software and fitted to a model for a bivalent analyte with Chi square values less than 1 in each case.

4.5.5 gp120 binding by ELISA

A primary HIV-1 B clade isolate termed D117III [28] was immobilized on microtiter plates overnight at 4 °C. After three washes with TBS/Tween, recombinant sCD4 in PBS (gift of R. Sweet, SmithKline Beecham, obtained through the AIDS Reagent Program) was added at 10 ng/ml, 1 ng/ml and nil. Plates were left at room temperature for 5 h, supernatant was aspirated and complexes were fixed and inactivated with 4 % paraformaldehyde for 45 min. Wells were washed as before and blocked with MP/PBS/Tween for 2 h. After further washes, B ~ P3A9/1, B ~ P5C7/1 and B ~ only (see Sect. 4.4) were added at 100, 10 and 1 μg/ml and no peptide in MP/PBS/Tween. Plates were left at 4 °C overnight and washed; bound peptide was detected using a streptavidin-HRP conjugate (Immunotech, Marseille, France) diluted 1/500 in PBS. After 2 h at room temperature and washed as above, plates were developed with o-phenylenediamine (OPD) substrate (Sigma chemicals). The reaction was stopped after 6 min with an equal amount of 1N H_2SO_4. A was measured at 495 nm (reference: 620 nm).

4.6 Indirect immunofluorescence

The murine prelymphoma B cell line L1.2 stably transfected with and expressing CCR5 at high levels was cultured [21] and cells were immobilized on poly-L-lysine-coated microscope slides by cytospin and air dried, before addition of 50 μl mAb 3A9 or 5C7 (5 μg/ml in PBS with 1 % BSA). Cells were exposed to FITC-conjugated anti-mouse antibody (Silenus) at 1/2000 in 0.5 M carbonate buffer with 1 % BSA, pH 8.6. The slides were dried and mounted using 90 % glyc-

erol, 10% PBS and 0.1% p-phenylenediamine, pH 8.0. Epifluorescent microscopy was performed using a 40 × objective lens. Images were digitized and emitted fluorescence was measured in OD units using MCID image analysis software (Imaging Research Inc., Catharines, ON, Canada). Background fluorescence was subtracted. Peptide P3A9/1c was added at concentrations of 100, 20, 10 and 5 μg/ml, or no peptide, to the primary antibody before exposure to the cells and epifluorescence microscopy, to assess inhibition of binding of mAb to CCR5.

4.7 Statistics

Data were tested for significance using Student's *t*-test.

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References

1 **Murphy, P. M.,** The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol.* 1994. **12:** 593–633.

2 **Baggiolini, M., Dewald, B. and Moser, B.,** Human chemokines: an update. *Annu. Rev. Immunol.* 1997. **15:** 675–705.

3 **Baggiolini, M.,** Chemokines and leukocyte traffic. *Nature* 1998. **392:** 565–568.

4 **Gale, L. M. and McColl, S. R.,** Chemokines: extracellular messengers for all occasions? *Bioessays* 1999. **21:** 17–28.

5 **Locati, M. and Murphy, P. M.,** Chemokines and chemokine receptors: biological and clinical relevance in inflammation and AIDS. *Annu. Rev. Med.* 1999. **50:** 425–440.

6 **Lee, B. and Montaner, L. J.,** Chemokine immunobiology in HIV-1 pathogenesis. *J. Leukoc. Biol.* 1999. **65:** 552–565.

7 **Henderson, R., Baldwin, J. M., Ceska, T. A., Zemblin, F., Beckmann, E. and Downing, K. H.,** Model for the structure of bacteriorhodopsin based on high-resolution electron cryomicroscopy. *J. Mol. Biol.* 1990. **213:** 899–929.

8 **Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A.,** HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein coupled receptor. *Science* 1996. **272:** 872–877.

9 **Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J.,** The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 1996. **85:** 1135–1148.

10 **Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. and Berger, E. A.,** C CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996. **272:** 1955–1958.

11 **Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marman, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. and Landau, N. R.,** Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996. **381:** 661–666.

12 **Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. and Paxton, W. A.,** HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996. **381:** 667–673.

13 **Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G. and Doms, R. W.,** A dual-tropic primary HIV-1 isolate that uses fusion and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 1996. **85:** 1149–1158.

14 **Clapham, P. R. and Weiss, R. A.,** Immunodeficiency viruses. Spoilt for choice of co-receptors. *Nature* 1997. **388:** 230–231.

15 **Moore, J. P., Trkola, A. and Dragic, T.,** Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* 1997. **9:** 551–562.

16 **He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J. and Gabuzda, D.,** CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* 1997. **385:** 645–649.

17 **Berger, E. A., Doms, R. W., Fenyö, E.-M., Korber, B. T. M., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J. and Weiss, R. A.,** A new classification for HIV-1. *Nature* 1998. **391:** 240.

18 **Berger, E. A., Murphy, P. M. and Farber, J. M.,** Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism and disease. *Annu. Rev. Immunol.* 1999. **17:** 657–700.

19 **Zhang, L., He, T., Huang, Y., Chen, Z., Guo, Y., Wu, S., Kunstmann, K. J., Brown, R. C., Phair, J. P., Neumann, A. U., Ho, D. D. and Wolinsky, S. M.,** Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. *J. Virol.* 1998. **72:** 9307–9312.

20 **Li, S., Juarez, J., Alali, M., Dwyer, D., Collman, R., Cunningham, A. and Naif, H. M.,** Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. *J. Virol.* 1999. **73:** 9741–9755.

21 **Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R. A. and Mackay, C. R.,** CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J. Exp. Med.* 1997. **185:** 1681–1692.

22 **Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S. and Landau, N. R.,** Change in coreceptor use correlates with disease progression in HIV-1 infected individuals. *J. Exp. Med.* 1997. **185:** 621–628.

23 **Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G. and Parmentier, M.,** Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 1996. **382:** 722–725.

24 **Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A. and Landau, N. R.,** Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 1996. **86:** 367–377.

25 **Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C. and Lusso, P.,** Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* 1995. **270:** 1811–1815.

26 **Sommons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Bruser, R., Wells, T. N. C. and Proudfoot, A. E.,** Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 1997. **276:** 276–279.

27 **Wu, L., LaRosa, G., Kassam, N., Gordon, C. J., Heath, H., Ruffing, N., Chen, H., Humblias, J., Samson, M., Parmentier, M., Moore, J. P. and Mackay, C. R.,** Interactions of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine receptor binding. *J. Exp. Med.* 1997. **186:** 1373–1381.

28 **Ruebsamen-Waigmann, H., Willems, W. R, Bertram, U. and von Briesen, H.,** Reversal of HIV-phenotype to fulminant replication on macrophages in perinatal transmission. *Lancet* 1989. **11:** 1155–1156.

29 **Samson, M., Labbe, O., Mollerreau, C., Vassart, G. and Parmentier, M.,** Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemsitry* 1996. **35:** 3362–3367.

30 **Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M. and Doms, R. W.,** Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreptor function. *J. Biol. Chem.* 1999. **274:** 9617–9626.

31 **Olson, W. C., Rabut, G. E. E., Nagashima, K. A., Tran, D. N. H., Anselma, D. J., Monard, S. P., Segal, J. P., Thompson, D. A. D., Kajumo, F., Guo, Y., Moore, J. P., Maddon, P. J. and Dragic, T.,** Differential inhibition of human immunodeficiency virus type 1 fusion, gp120, and CC-chemokine activity by monoclonal antibodies to CCR5. *J. Virol.* 1999. **73:** 4145–4155.

32 **Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Smyth, R. J., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W. and Parmentier, M.,** Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell* 1996. **87:** 437–446.

33 **Choe, H., Martin, K. A., Farzan, M., Sodroski, J., Gerard, N. P. and Gerard, C.,** Structural interaction between chemokine receptors, gp120 and CD4. *Semin. Immunol.* 1998. **10:** 249–257.

34 **Genoud, S., Kajumo, F., Guo, Y., Thompson, D. and Dragic, T.,** CCR5-mediated human immunodeficiency virus entry depends on an amino-terminal gp120-binding site and the conformational integrity of all four extracellular domains. *J. Virol.* **73:** 1645–1648.

35 **Farzan, M., Choe, H., Vaca, L., Martin, K., Sun, Y., Desjardinns, E., Ruffing, N., Wu, L., Wyatt, R., Gerard, C. and Sodorski, J.,** A tyrosine-rich region in the N terminus od CCR5 is important for human immunodeficiency virus type 1 entry and mediates and association between gp120 and CCR5. *J. Virol.* 1998. **72:** 1160–1164.

36 **Ross, T. M., Bieniasz, P. D. and Cullen, B. R.,** Multiple residues contribute to the inability of murine CCR-5 to function as a coreceptor for macrophage-tropic human immunodeficiency virus type 1 isolates. *J. Virol.* 1998. **72:** 1918–1924.

37 **Doranz, B. J., Lu, Z.-H., Rucker, J., Zhang, T.-Y., Sharron, M., Cen, Y.-H., Wang, Z.-X., Guo, H.-H., Du, J.-G., Accavitti, M. A., Doms, R. W. and Peiper, S. C.,** Two distinct CCR5 domains can mediate co-receptor usage by human immunodeficiency virus type 1. *J. Virol.* 1997. **71:** 6305–6314.

38 **Rabut, G. E. E., Konner, J. A., Kajumo, F., Moore, J. P. and Dragic, T.,** Alanine substitutions of polar and nonpolar residues in the amino-terminal domain if CCR5 differently impair entry of macrophage- and dual-tropic isolates of human immunodeficiency virus type1. *J. Virol.* 1998. **72:** 3464–3468.

39 **Dragic, T., Trkola, A., Lin, S. W., Nagahima, K. A., Kajumo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P. and Maddon, P. J.,** Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type1 entry. *J. Virol.* 1998. **72:** 279–285.

40 **Berson, F. B. and Doms, R. W.,** Structure-function studies of the HIV-1 coreceptors. *Semin. Immunol.* 1998. **10:** 237–248.

41 **Alkhatib, G., Ahuja, S. S., Light, D., Mummidi, S., Berger, E. A. and Ahuja, S. K.,** CC chemokine receptor 5-mediated signaling and HIV-1 co-receptor activity share common structural determinants. *J. Biol. Chem.* 1997. **272:** 19771–19776.

42 **Kwong, P. D., Wyatt, R., Robinson, J., Sweets, R. W., Sodroski, J. and Hendrickson, W. A.,** Structure of an HIV-1 gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing antibody. *Nature* 1998. **393:** 648–659.

43 **Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A. and Sodroski, J.,** A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* 1998. **280:** 1949–1953.

44 **Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R.,** Mimicking of discontinuous epitopes by phage-displayed peptides, I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene* 1993. **128:** 51–57.

45 **Parmley, S. F. and Smith, G. P.,** Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* 1988. **73:** 305–318.

46 **Sambrook, J., Fritsch, E. F. and Maniatis, T.** (Eds.) *Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Habor Laboratory Press, Cold Spring Habor 1989.

47 **Fields, G. B. and Noble, R. L.,** Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pet. Protein Res.* 1990. **35:** 161–214.

48 **Holmdahl, R., Rubin, K., Klareskog, L., Larsson, E. and Wigzell, H.,** Characterization of the antibody response in mice with type II collagen induced arthritis, using monoclonal anti-type II collagen antibodies. *Arthritis Rheum.* 1986. **29:** 400–410.

49 **Chang, Y. C. and Gottlieb, D. L.,** Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J. Neurosci.* 1988. **8:** 2123–2130.

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Note added in proof: Zhou et al. recently published a protein structure modeling and site-directed mutagenesis study indicating that N-terminal residues (Y10, K26 particularly) as well as residues from the second extracellular loop are involved in chemokine binding and signaling: Zhou, N., Luo, Z., Hall, J. W., Luo, J., Han, X. and Huang, Z., *Eur. J. Immunol.* 2000, **30:** 164–173.